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The Effects of Amino Acids and Ammonium on the Growth of Plant Cells in Suspension Culture

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ABSTRACT

A suspension culture of soybean (*Glycine max* L.) was grown on a defined medium in which the nitrogen sources were nitrate (25 mM) and ammonium (2 mM). The cells did not grow on nitrate unless the medium was supplemented with ammonium or glutamine. The L- and D-isomers of 12 amino acids tested singly could not replace ammonium. Most amino acids (4 mM) inhibited growth when the cells were cultured on nitrate and ammonium. Cells from five other plants (*Reseda luteoli* L.; *Triticum monococcum* L.; flax, *Linum usitatissimum* L.; horseradish, *Amoracia lapathifolia* Gilib; *Haplopappus gracilis* L.) grew on the defined medium with nitrate (25 mM) as the sole nitrogen source. Higher cell yields were obtained when ammonium (2 mM) or glutamine also was present. Supplementing the defined medium with high concentrations of ammonium (20 mM) inhibited growth of soybean, *Haplopappus*, and wheat cells. Addition of citrate (5 mM) relieved the inhibitory effects of ammonium in soybean and wheat cells but not in the *Haplopappus* cells.

Plant cells cultured in liquid, defined media normally grow on nitrate or ammonium nitrate as the nitrogen source (3, 4, 8, 10, 16). Supplementing the media with mixtures of amino acids or commercial preparations of hydrolyzed proteins frequently enhances the growth (2, 14), although amino acids also may inhibit growth (5, 8).

The concentration of nitrogen and the relative amounts of ammonium and nitrate may be critical for growth and morphogenesis of plant cells (10, 15).

In an earlier report from this laboratory it was shown that a soybean cell culture growing on a defined medium required ammonium or glutamine in order to grow on nitrate (8). Growth ceased when nitrate was used without a source of reduced nitrogen. A series of amino acids has now been examined to determine their effectiveness as a replacement for ammonium sulfate. Furthermore, the effect of various nitrogen sources on the growth of soybean cells and cells of five other plants was investigated.

METHODS AND MATERIALS

Culture Conditions. The soybean cells were established from explants of soybean roots (*Glycine max* L. var. Mandarin) and maintained by serial subculturing for 5 years (6). Other cultures used in the experiments were: *Reseda luteoli* L.; wheat, *Triticum monococcum* L.; flax, *Linum usitatissimum* L.; and horseradish, *Amoracia lapathifolia* Gilib. These cultures were established according to methods described previously (6, 7). The *Haplopappus gracilis* culture was kindly supplied by Dr. T. Eriksson, University of Uppsala, Sweden. The composition of the basal medium is shown in Table I (8). The cells were grown in 250-ml DeLong flasks in a total volume of medium and inoculum of 40 ml. The flasks were incubated at 27 C in continuous light on a gyrotory shaker (6).

The amino acids used in the experiments were filter-sterilized and added to the autoclaved medium.

Analytical Methods. For determining dry weight, the cells were collected on Miracloth, washed with water, and dried in a vacuum oven at 60 C for 20 hr. Total protein was determined by a micro-Kjeldahl method.

RESULTS

The results in Table II serve to illustrate that the lack of growth of soybean cells in nitrate alone is not influenced by the concentration of nitrate. The other important observation is the profound influence of ammonium on the utilization of nitrate reflected in the increased growth rate. Ammonium is only one form of reduced nitrogen, and other sources were subsequently tested. In a series of experiments a number of amino acids were added one at a time at a concentration of 1, 2, and 4 mM. These concentrations were selected since those were the levels at which ammonium sulfate was used. Cell growth was reduced in the presence of both isomers of most amino acids at the three concentrations. The data in Figure 1 show the effect on growth of the soybean cells when various amino acids were substituted for ammonium sulfate in the medium. The medium contained 25 mM nitrate, and the concentration of the amino acids was 4 mM. Addition of L-glutamine resulted in growth equal to that obtained with ammonium sulfate. The L-isomers of alanine and arginine could not replace ammonium, but the cell yield was higher than for cells grown in nitrate alone. Chromatographic analysis showed that the majority of the amino acids disappeared from the medium and were presumably absorbed.

The results in Figure 2 show the growth achieved by substituting glutamine, urea, and amino acids related to the urea cycle for ammonium sulfate. The L-glutamine was fully equal to ammonium sulfate in supporting growth. On the other hand, the D-isomer did not support growth, which may partly be due to an inability of the cells to absorb D-glutamine. When ornithine, citrulline, or urea was added, the growth was less than, or equal to, that obtained when nitrate was used alone. Urea was later tested at concentrations of 1, 2, 4, and 8 mM, and in all cases the cell yield did not exceed that obtained with nitrate alone.

The lack of growth in the presence of individual amino acids

could be due to the inability of the compounds to substitute for ammonium or to inhibition of metabolic reactions in the cells.

These possibilities were further tested in experiments in which the cells were cultured in suboptimal amounts of ammonium. At a concentration of 0.25 mM ammonium sulfate the cells grew well but did not have adequate amounts of ammonium for maximum growth (Fig. 3). Individual amino acids were added together with 0.25 mM ammonium sulfate. Of the amino acids tested, only L-glutamine could supplement the ammonium salt. The L-isomer of glutamic acid was quite inhibitory, but addition of glutamine together with glutamic acid resulted in near normal growth. The amino acids were removed from the medium during incubation, suggesting that growth repression originated in the metabolic pathway at some point.

In order to test whether the requirement for ammonium or glutamine was unique for the soybean culture, several other cultures were tested (Table III). The cultures from *Reseda*, wheat, flax, and horseradish grew quite well on nitrate alone, but the yields were generally higher when nitrate was supplemented with ammonium or glutamine. Wheat, however, did not benefit to the same degree from the presence of ammonium, but glutamine enhanced growth in the same manner as observed for *Reseda*, flax, and horseradish cells. These results indicated that ammonium was not essential for growth by the four cultures, although there was some stimulation of growth of *Reseda*, flax, and horseradish when ammonium sulfate was added.

In another experiment a new culture of soybean (soybean-2) was compared with cells of wheat, *Haplopappus*, and the original soybean culture. The growth rate of the soybean-2 culture was

Table II. *Effect of Ammonium on Utilization of Nitrate by Soybean Cells in Suspension Culture*

The inoculum was 19 mg and the growing period was 6 days.

Concentration			Final Dry Weight
KNO_3	KCl	$(NH_4)_2SO_4$	
mM			mg
5	20	0	35
10	15	0	35
20	5	0	35
25	0	0	36
5	20	1	26
10	15	1	114
20	5	1	175
25	0	1	180

Table I. *Composition of B5 Medium for Suspension Cultures of Plant Cells*

The pH of the medium was 5.5.

Compound	Concentration	Compound	Concentration
	mg/liter		mg/liter
$NaH_2PO_4 \cdot H_2O$	150	$MnSO_4 \cdot H_2O$	10
KNO_3	2500	H_3BO_3	3
$(NH_4)_2SO_4$	134	$ZnSO_4 \cdot 7\ H_2O$	2
$MgSO_4 \cdot 7\ H_2O$	250	$Na_2MoO_4 \cdot 2\ H_2O$	0.25
$CaCl_2 \cdot 2\ H_2O$	150	$CuSO_4$	0.025
Iron[1]	28	$CoCl_2 \cdot 6\ H_2O$	0.025
Nicotinic acid	1	KI	0.750
Thiamine·HCl	10	Sucrose	20,000
Pyridoxine·HCl	1	2,4-D	1
Myoinositol	100		

[1] Sequestrene 330 Fe obtained from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, New York.

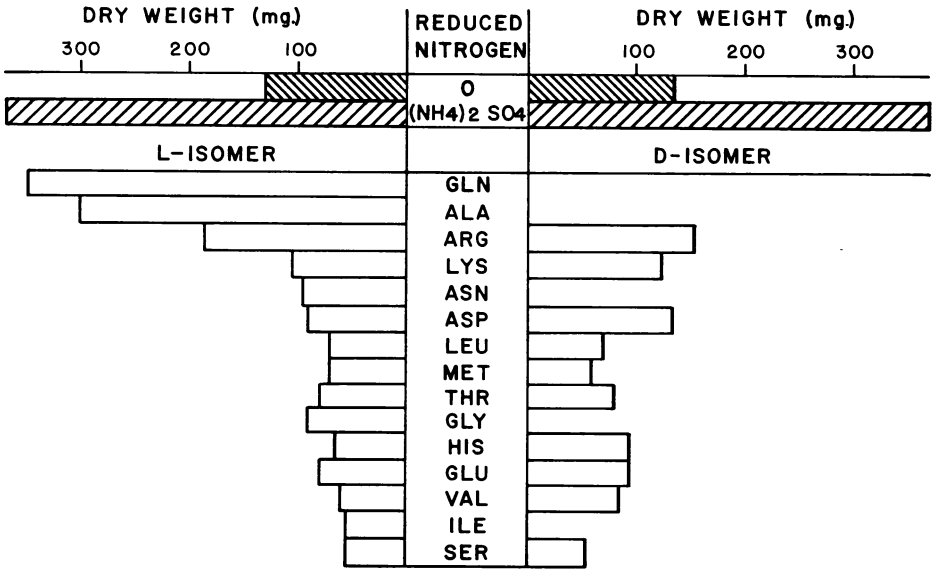

FIG. 1. The effect of D- and L-isomers of amino acids on the growth of soybean cells in suspension culture. The inoculum was 34 mg, and the growing period was 5 days. The compounds added were ammonium sulfate, 1 mM; and amino acids, 4 mM (filter-sterilized).

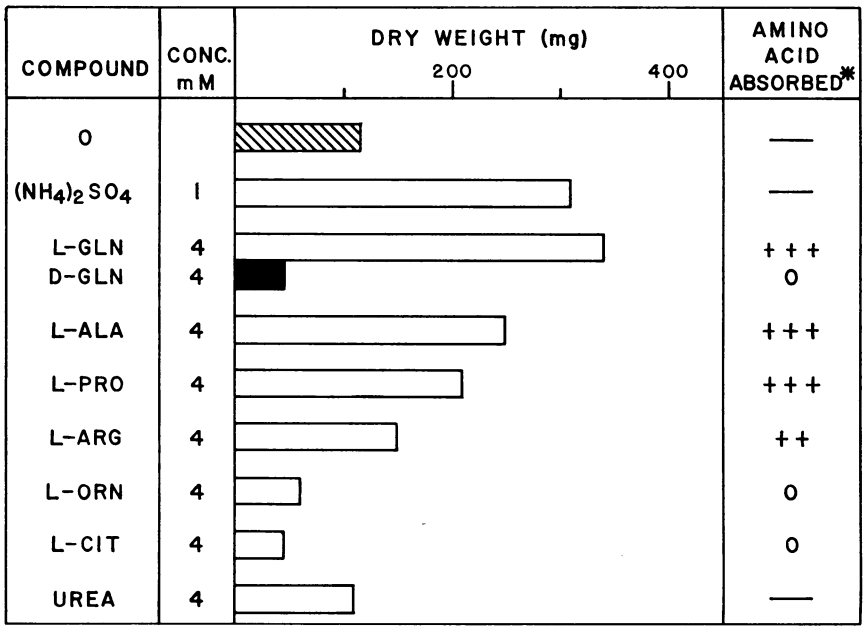

FIG. 2. The effect of various sources of reduced nitrogen on the growth of soybean cell cultures. *: Estimated by paper chromatography; +++: amino acid completely absorbed. Inoculum was 20 mg, and the growing period 6 days.

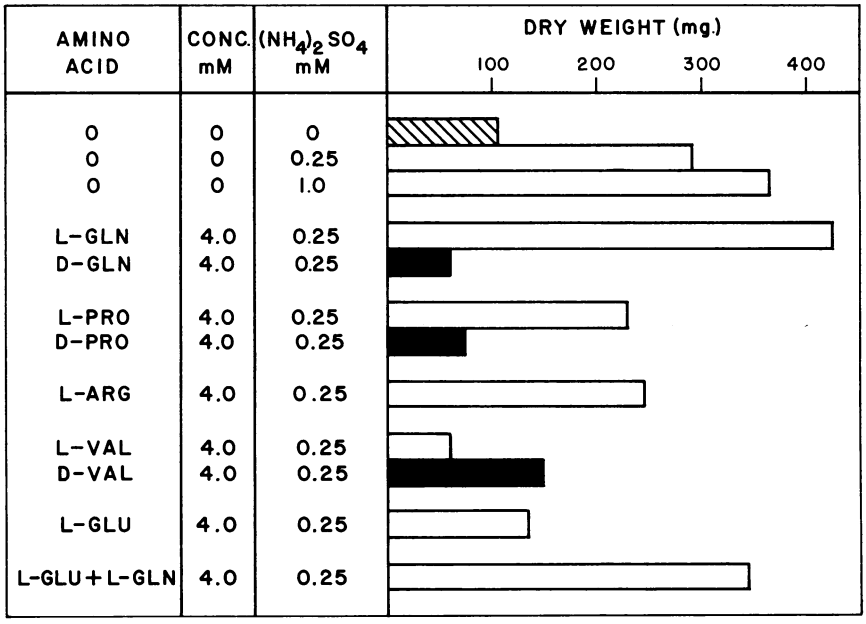

FIG. 3. The effect of amino acids on the growth of soybean cells cultured in suboptimal amounts of ammonium. The culture period was 6 days, and the size of the inoculum was 25 mg.

relatively low (Table IV). The two soybean cultures grew poorly on nitrate alone while low concentrations of ammonium enhanced the growth of both cultures. The *Haplopappus* and wheat cells grew well on the nitrate medium. All cultures were inhibited by 20 mM ammonium. Addition of citrate resulted in high yields of soybean-1 and wheat cells, while further repressing the growth of soybean-2 and the *Haplopappus* cells.

DISCUSSION

Plants normally use nitrate as the source of nitrogen, and it could be expected that cultures of plant cells would have no difficulty in utilizing nitrate for their nitrogen supply. The soybean cells appear to have a special requirement for reduced nitrogen to enable them to grow on nitrate. Ammonium and glutamine satisfy this requirement most adequately. Most amino acids inhibited growth and could not be substituted for ammonia. Filner (5) grew tobacco cells in a medium in which nitrate was the only source of nitrogen. He found that some amino acids inhibited growth and also repressed nitrate reductase.

Ammonium is a ready source of reduced nitrogen, and possibly is absorbed as the amine (1). It can be used as an alternative to glutamine in the formation of nucleotides (13) and indole (9), but glutamine usually is preferred in the enzyme reactions. The enhancement of growth by low concentrations of ammonium appears to be a characteristic of the soybean cells and indicates that

Table III. *Cell Yield of Cultures from Several Plant Species Grown in Defined Medium in Various Nitrogen Sources*

Culture	Inoculum	Nitrogen Source[1]			Growing Period
		Nitrate	Nitrate + ammonium sulfate	Nitrate + glutamine	
	mg dry wt				*days*
Reseda[2]	34	183	215	414	10
Reseda[2]	50	181	249	405	10
Reseda	16	173	386	415	11
Wheat	45	146	142	254	10
Wheat	80	238	290	305	6
Wheat	41	226	211	302	10
Flax embryo	25	160	302	211	10
Flax embryo	15	127	356	361	11
Horseradish	20	132	181	226	10

[1] The concentration of nitrate was 25 mM; ammonium sulfate, 1 mM; and L-glutamine, 4 mM.

[2] The inoculum was cells grown in a casein-hydrolysate medium.

Table IV. *Effect of Nitrogen Source on the Growth of Suspension Cultures of Plant Cells*

Growth is expressed as mg dry weight.

Nitrogen Source and Concn	Growth of Cultures[1]			
	Soybean-1 (24 mg, 5 days)	Soybean-2 (33 mg, 13 days)	*Haplopappus* (30 mg, 7 days)	Wheat (53 mg, 7 days)
	mg dry wt			
KNO_3	69	144	298	311
KNO_3(25 mM) + $(NH_4)_2SO_4$(1 mM)	305	310	258	390
KNO_3(25 mM) + NH_4Cl(20 mM)	155	108	145	113
KNO_3(25 mM) + NH_4Cl(20 mM) + citrate(5 mM)	384	84	96	385

[1] Given in parentheses: Milligrams dry weight of inoculum and growing period in days.

the reduction of nitrate to the level of ammonium in these cells is in some way stimulated by ammonium.

The reduction in growth of all cell cultures by high concentrations of ammonium may reflect ammonium inhibition of enzyme systems. Ammonium inhibits glutamine synthetase in *Escherichia coli* (11) and causes a decrease in the levels of reduced pyridine nucleotides in liver mitochondria (12). The latter is considered to be an inhibition of isocitrate dehydrogenase. High concentrations of ammonium may have a similar effect on the tricarboxylic acid cycle in plant cells and thus limit the formation of keto acids required for amino acid synthesis and transamination. The observed increase in growth of the cells in high concentrations of ammonium supplemented by citrate may be the result of a stimulation of the tricarboxylic acid cycle and also an increase in the levels of reduced pyridine nucleotides required for the formation of glutamic acid. Recent results indicate that succinate and malate similarly overcome the inhibition by ammonia. The stimulation by citrate was observed only in soybean-1 and wheat. These cell lines have been cultured for several years. The soybean-2 and the *Haplopappus* cells had been grown for only a few months. Thus, the difference in response of the two sets of cultures suggests a distinct difference in metabolic patterns of the cells.

The enhancement of the growth rate by glutamine could be explained on the basis that glutamine provided a readily available source of nitrogen, the implication being that the formation of the necessary carbon skeleton or the reduction of nitrate to ammonium is a limiting factor in the cells. Alternatively, the improved growth may be due to a higher concentration of nitrogen. The soybean cells appeared to absorb nitrate fairly quickly, with the result that the ambient nitrate concentration would be reduced. Addition of glutamine, which is relatively nontoxic (8), would enable the cells to maintain a high growth rate for a longer period. A more accurate assessment of any direct benefit from glutamine would be possible by culturing the cells in a system in which the nitrogen could be replenished continuously.

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